OVEREXPRESSION OF TYRO3 AND ITS IMPLICATION ON HEPATOCELLULAR CARCINOMA (HCC) PROGRESSION

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TABLE OF CONTENTS

Contents **Page**

SUMMARY

The lack of effective treatment against hepatocellular carcinoma (HCC), the fifth most common malignancy and the third leading cause of cancer deaths worldwide calls for direct efforts to better understand the disease and identify new drug targets. In the search for novel multi-kinase inhibitors to treat this disease, our group found a very potent compound which acts on a relatively uncharacterized receptor tyrosine kinase, Tyro3. To explore the potential role of tyrosine kinase Tyro3 in HCC, we examined the expression of Tyro3 in HCC tumors and correlated it with clinical outcomes. Using cDNAs derived from 56 HCC patients and quantitative RT-PCR (qRT-PCR) analysis of Tyro3, we found frequent and significant overexpression which also corresponded to elevation of clinico-pathological markers for HCC such as hepatitis B virus (HBV) infection, α-fetoprotein (AFP), aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels. To determine the cause-and-effect relationship between Tyro3 expression and these HCC phenotypes, we performed *in vitro* investigation using siRNA silencing of Tyro3 in a high expressing HCC cell line, Hep3B and found it to suppress cell proliferation. From these efforts, we have gathered important basis for further work to characterize Tyro3 as a potential and novel drug target in HCC. Of equal importance, we have successfully developed useful and relevant *in vitro* models that will support subsequent effort to understand the exact mechanism behind its effect.

LIST OF TABLES

LIST OF FIGURES

ABBREVIATION LIST

ADH	Alcohol Dehydrogenase
AFB1	Aflatoxin B1
AFP	α-fetoprotein
ALDH	Acetaldehyde Dehydrogenase
ALT	Alanine Aminotransferase
APS	Ammonium persulfate
AST	Aspartate Aminotransferase
ATCC	American Type Culture Collection
ATP	Adenosine Triphosphate
BCA	Bicinchoninic Acid
DEPC	Diethylpyrocarbonate
DMEM	Dulbecco's Modified Eagle's Medium
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic Acid
EGFR	Epidermal Growth Factor Receptor
ERK	Extracellular Signal-regulated Kinase
FAK	Focal Adhesion Kinase
FBS	Fetal Bovine Serum
FCS	Fetal Calf Serum
FDA	Food and Drug Administration
FGFR1	Fibroblast Growth Factor Receptor 1
FGFR4	Fibroblast growth factor receptor 4

FNIII	Fibronection Type III
GIST	Gastrointestinal Stromal Tumors
HBV	Hepatitis B Virus
HCC	Hepatocellular Carcinoma
HCV	Hepatitis C Virus
HRP	Horseradish Peroxidase
IGF1R	Type1 insulin-like growth factor receptor
IGF-2	Insulin-like growth factor 2
JAK	Janus Kinase
MAPK	Mitogen-activated Protein Kinase
MEM	Minimal Essential Medium
MITF	Microphthalmia-associated Transcription Factor
MITF-M	Melanocyte-specific MITF
mTOR	Mammalian Target of Rapamycin
mTORC1	Mammalian Target of Rapamycin Complex 1
mTORC2	Mammalian Target of Rapamycin Complex 2
NEAA	Non-essential Amino Acids
NSCLC	non-small cell lung carcinomas
NUH	National University Hospital
PBS	Phosphate-buffered Saline
PCR	Polymerase Chain Reaction
PDGFR	Platelet-derived Growth Factor Receptor
PI3K	Phosphoinositide 3-kinase
PLC	Primary Liver Cancer
PP1	Protein Phosphatase 1

PVDF	Polyvinylidene Difluoride
R&D	Research and Development
RLU	Relative Luminescence Unit
RT-PCR	Real-time Polymerase Chain Reaction
SDS-PAGE	Sodium Dodecyl Sulfate-polyacrylamide Gel Electrophoresis
TACE	Transarterial Chemoembolization
TAE	Tris- acetate- EDTA
TEMED	N,N,N',N'-tetra methylene diamine
VEGF	Vascular Endothelial Growth Factor
VEGFR	Vascular Endothelial Growth Factor Receptor
VEGFR2	Vascular endothelial growth factor receptor 2
YAP	Yes-associated Protein

1.0 INTRODUCTION

1. 1 Introduction to HCC

Primary liver cancer is the fifth most common cancer type worldwide. Among which, hepatocellular carcinoma (HCC) contributes about 85-90% of all cases [1]. In 2008, there were about 696,000 deaths, making this the 3rd largest cause of cancer death and hence a major healthcare burden [2]. While the disease inflicts people of all ethnicities, people in Asian countries are reported to have higher incidence of HCC than the Western countries [3]. Furthermore, gender difference exists significantly whereby males represent higher risk of developing HCC than females, especially for patients who are older than 50 years old. The ratio of male to female for HCC ranges from 2:1 to 4:1[1]. For this reason, HCC is the 4th most frequently occurring cancer in male and ranks much lower for the female gender [2]. The exact mechanism for such differences is not clear but it was suggested that the differential expression of androgen receptors may correlate with this gender disparity [4]. HCC also exhibits age disparity whereby risk and manifestation increase significantly with age. For example, after liver transplantation, the immune response for older people is likely to be much higher than younger patients. These observations may also underline the prolonged incubation period before disease manifestation. Hence, associating these epidemiological trends to etiology will be necessary.

1.2 Etiology of HCC

Like several other malignancies, HCC is a disease with multiple causes. To date the main causes that have been identified include chronic alcohol abuse, hepatitis C infection (HCV), hepatitis B infection (HBV), and exposure to chemical carcinogens (such as Aflatoxins and vinyl chloride). There is also significant geographical disparity in terms of the etiology of HCC. In US, HCV infection is the predominant cause of HCC, whereas in China, HBV is most prevalent. Together, HBV and HCV account for 80%-90% of all HCC incidences worldwide [3]. Both diseases are chronic infection of the liver and carriers of these viruses can remain asymptomatic for 30-50 years before some of them progress into HCC. Effective vaccination program for HBV started in the 1980s and such measures are believed to tremendously reduce the incidence of HCC in time to come [1]. However, for chronic HBV and HCV carriers who were already infected prior to vaccination program, their risk profile remains high and hence, it is speculated that this disease will continue to be a healthcare menace for many more years to come.

Chronic alcohol abuse can lead to alcoholic liver formation, which also predisposes the individual to HCC. Alcohol is metabolized to acetaldehyde by alcohol dehydrogenase (ADH) and subsequently to acetic acid by acetaldehyde dehydrogenase (ALDH). Acetaldehyde is a known chemical carcinogen that can react with proteins/nucleic acids as a result of its electrophilicity. In this context, persistent exposure to acetaldehyde due to chronic alcoholism or failure in metabolism can result in a liver-directed injury that often begins with fatty liver (steatosis) and liver fibrosis. Unresolved cirrhosis will then predispose the individual to the more detrimental consequences of hepatocarcinogenesis [5, 6].

Another major contributor of HCC arises from Aflatoxin B1 (AFB1) exposure. AFB1 is a mycotoxin produced by *Aspergillus flavus*. This fungus thrives in warm and moist environment and populates in crops such as peanuts, corns and maize etc. When ingested as part of the contaminated food product, AFB1 is metabolized by Cytochrome P450 enzymes via oxidation to generate reactive epoxides that subsequent bind and modify DNA [7]. AFB1 has in fact been shown to be one of the most potent carcinogens known to date. Today, AFB1 is a major contributor to HCC particularly in Southern China and the Sub-Saharan continent where moist and humid conditions aggravate the contamination of the abovementioned food products.

1.3 Hepatocarcinogenesis

Regardless of the source of the initial lesion in the liver (i.e. HBV, HCV infection, alcoholic liver injury, aflatoxin etc), some common processes in hepatocarcinogenesis emerge. The understanding of this process is pertinent to the development of effective strategies to cope with the problem. The disease usually follows a progression from a prolonged inflammatory state to eventually, tumorigenic development and progression. With persistent exposure to agents that cause liver inflammation, the injured liver activates compensatory responses that attempt to resolve the injury. This process involves the activation of hepatic stellate cells which increases the deposition of extracellular matrix such as collagen (fibrogenesis) [8]. This leads to an increase in scar tissue formation that compromises overall liver function. As the fibrotic condition aggravates, further reduction in hepatocellular function results in a liver cirrhotic state. Cirrhosis is a state of liver when liver tissue becomes more rigid, so that the normal function of liver such as detoxifying, glycogenesis and synthesis of clotting factors was decimated. This process can take

place over several years of incubation period. On average, it takes about 25-30 years to develop HCC after initial HCV exposure [1]. A similar hepatocarcinogenesis process was also reported for HBV infection.

The role of cirrhosis to HCC is especially critical as most HCC arise from cirrhosis as a pre-neoplastic event. Cirrhosis was described to accelerate hepatocarcinogenesis through various mechanisms. With chronic liver inflammation, hepatocytes reach their limits in regeneration due to the shortening of telomeres. This state of senescence will induce DNA damage leading to chromosomal instability [9]. Secondly, cirrhosis is also known to change the liver microenvironment due to the deposition of an altered extracellular matrix and increase oxidative stress that promotes tumor proliferation [10]. That said, there are yet patients with HBV infection who may directly develop HCC after a period of HCC infection without cirrhosis as a pre-neoplastic event [11]. The exact mechanistic details for the disease development await further investigation.

1.4 Current treatments for HCC

Today, treatment for HCC is suboptimal with 1-year and 3-year survival at approximately 20% and 5% respectively, and a median survival of only 8 months [12]. Most of the available treatment modalities are non-curative. Liver transplantation is the only curative approach. However, this option is only available for a small subset of patients who are presented with isolated and limited vascular invasion [13]. Moreover, the number of patients awaiting transplant far exceeds that of the number of genetically matching donors, hence transplantation is not a viable option for most patients. Post-transplantation, there is also a risk of organ rejection which reduces long term survival. Additionally, overall survival and disease-free

survival are also compromised by the presence of macroscopic vascular invasion and satellite nodules. Immune response also tends to be relatively high after liver transplantation. The 15-year survival is 58% in patients who undergo liver transplantation [14].

Besides complete liver transplantation, partial liver resection could help another subset of patients with isolated tumors and sufficient liver function. Together with transplantation, surgical resection may give an optimistic 5-year survival at about 5-60% [13]. Likewise, this option is not suited for the many patients who are first diagnosed at an advanced stage where intrahepatic metastasis has already occurred. Short of this option, other available treatments for HCC now include locoregional therapies such as ethanol injection, radiofrequency ablation, and transarterial chemoembolization (TACE). Also, there is a place for conventional chemotherapy and more recently, molecular targeted drug therapies are introduced but they are mostly in the experimental phase [12]. Ethanol injection is suitable for small and single tumors, but the average survival rate is relatively low [15]. Radiofrequency ablation is also used to treat HCC, especially in combination with hepatic resection. But it also suffers from some limitations such as biliary tract damage, liver failure and local recurrence [16].

Systemic chemotherapy has limited role in HCC as it is often left as a final line of action. At this stage, patients are almost resistant to available conventional chemotherapy, even though some other malignancies may have responded more positively to such treatments. Furthermore, these agents suffer from common side effects due to non-targeted cytotoxicities on rapidly dividing cells such as hair loss, fatigue and immunosuppression. For example, doxorubicin and cisplatin are both used systemically as chemotherapeutic agents to treat HCC but neither

showed significant survival advantage. Overall survival was further reduced for patients with high HBV DNA load [17]. An additional challenge comes from the fact that HCC is often manifested as a dual disease of both cancer and liver dysfunction due to the underlying cirrhosis. Hence, many pharmacological interventions (particularly those that are extensively metabolized by the liver) will experience abnormal pharmacokinetics that requires specialized care and monitoring. Clearly, there are several limitations for existing treatment for hepatocellular carcinoma. Therefore, this generates a dire need for new methods for HCC treatment that could benefit more patients, reduce immune response and prolong patients' survival rate and survival time

1.5 New drugs for HCC treatment

In view of these challenges, newer molecular targeted therapies that recently emerged in the market for other malignancies are being explored for HCC [18]. Molecular targeted therapy is a revolutionary approach to cancer treatment by targeting the underlying mechanism that drives the cancer phenotypes. These phenotypes can include that of hyperproliferation, anti-apoptosis, cellular transformation, angiogenesis and even inflammation [19]. It is believed that every cancer arises from some cell signaling aberrations that may be different from one cancer to another. Therefore, it is possible to identify such aberrations and block them selectively so that the effect on adjacent normal cells will be minimized. This overcomes the challenge of cytotoxicities on other hyperproliferating normal tissues on which conventional chemotherapy tends to exert. Furthermore, the specific inhibition of cancer phenotypes can also lead to a more wholesome resolution of the disease, besides just eliminating the rapidly dividing cancer cell population. For

example, bevacizumab, a humanized anti-vascular endothelial growth factor (VEGF) monoclonal antibody that binds and neutralizes human VEGF, was approved in 2004 for first-line treatment of metastatic colorectal cancer patients in combination with 5-fluorouracil-based chemotherapy [20]. VEGF is an important growth factor that binds and activates vascular endothelial growth factor receptor (VEGFR), which is found to be overexpressed in several colorectal carcinomas to support neo-angiogenesis and the growth of the tumor. Hence, its inhibition is aimed to block new blood vessels to deprive the tumor of the necessary nutrients to support further spreading of the cancerous growth. Gefitinib can target the epidermal growth factor receptor (EGFR) tyrosine kinase and is approved in the U.S. for non small cell lung cancer. Several non-small cell lung carcinomas (NSCLC) carry activating mutation of EGFR which results in constitutively active signaling of downstream extracellular signal-regulated kinase (ERK) signaling to support cancer cell proliferation [21]. Hence, this agent specifically corrects the aberration only in the cancer tissue that manifests this genetic lesion.

Sorafenib is a drug approved by the Food and Drug Administration (FDA) in year 2007 for HCC treatment. It is also used for the treatment of renal cell carcinoma. This agent became the very first molecular target therapy that opened new possibilities for the treatment of HCC. In the hallmark paper leading to its approval, an international phase III, placebo-controlled study showed that sorafenib significantly improved overall survival (median overall survival 10.7 months with sorafenib vs. 7.9 months with placebo) [22]. In a follow-up clinical trials focusing on Asian population, the median overall survival for the sorafenib arm is 6.5 months vs. 4.2 months for placebo [23]. The mechanism underlying sorafenib treatment is that this tyrosine kinase inhibitor is able to block several signaling pathways, resulting in

repression of cell proliferation [24]. Sorafenib was originally developed as a RAF kinase inhibitor which then blocks ERK signaling pathway and alters some phenotypes of liver cancer cell lines. More recently, sorafenib was shown to exhibit multi-targeting effect and exert inhibitory effect on some tyrosine kinases. The regulation of angiogenesis is a complex, multistep process resulting from a dynamic balance between pro-angiogenic and anti-angiogenic factors. Two of the most important regulators of this process are the VEGF and platelet-derived growth factor receptor (PDGFR) [25]. It was subsequently evaluated that these tyrosine kinase targets could turn out to be more critical for its efficacy as anti-cancer agent, particularly in metastatic tumor.

The unprecedented success of sorafenib turned the page on pharmacotherapy against HCC as it triggered further investigation into other tyrosine kinase inhibitors in the treatment of HCC. The benefit of this approach is believed that tyrosine kinase inhibitors can target those tyrosine kinases that are important in the initiation and progression of HCC. This is an emerging field as more and more tyrosine kinases are being identified to be implicated in HCC.

1.6 Tyrosine kinases implicated in HCC

Therefore, a successful application of tyrosine kinase inhibitors requires a clear understanding of their involvement in the target disease. Generally, aberrant signaling of tyrosine kinases are mediated by increased activity either mediated by increased binding of growth factors, overexpression of the receptors, or mutation of the receptor or their downstream signaling targets to result in elevated cellular activities and responses. For receptor tyrosine kinases, they are usually activated by first binding to specific ligands (usually growth factors). This results in

conformational change which allows the dimerization of the receptor and the activation of the intracellular kinase domain. The innate kinase activity results in transphosphorylation of the receptor itself or other kinase targets which generates anchoring sites for other cell signaling molecules that subsequently activate a cascade of pathways resulting in cancer-like behaviors. Many of their signaling converge onto pathways such as phosphoinositide 3-kinase (PI3K) and ERK signaling which are important in mediating cell cycling, proliferation, cell invasion, and metastatic behavior, apoptosis as well as other cell phenotypes which attribute partially to their suitability as anti-cancer targets [26, 27].

For instance, activation of EGFR and type1 insulin-like growth factor receptor (IGF1R) tyrosine kinases mediate the activation of PI3K [28, 29]. This in turn will elevate Akt phosphorylation which is then responsible for inhibiting mitochondrial anti-apoptotic mechanism through BAD and BCL-x_L, as well as mTOR, which promotes cell proliferation. The mammalian target of rapamicin mammalian target of rapamycin (mTOR) pathway is implicated widely in cancer pathophysiology. Dual inhibition of the mTOR kinase complexes, mammalian target of rapamycin complex 1 (mTORC1) and mammalian target of rapamycin complex 2 (mTORC2) decreases tumor xenograft growth in vivo [30]. Separately, ERK/mitogen-activated protein kinase (MAPK) pathway is another key regulator of cancer phenotypes such as proliferation, differentiation and even angiogenesis. When growth factors bind to various receptors such as EGFR, phosphorylation will trigger the association of adaptor molecules which in turn activate RAS/RAF/ERK signaling [31]. Therefore, the use of tyrosine kinase inhibitors such as gefitinib (EGFR inhibitor), was expected and have been shown to suppress some of these phenotypes [32].

With the essential role of the tyrosine kinases in cancer, another key that makes them suitable drug targets is their position in the top echelon of cellular signaling, as they generally bind to extracellular signaling molecules at their receptors and transmit this information intracellularly. Therefore, they are conveniently located for the binding of small molecules (<500 Daltons), and hence, highly druggable targets to consider for cancer therapeutics. Their popularity in pharmaceutical research and development (R&D) is further stimulated by early discoveries that several tyrosine kinases have already been reported to have implication on HCC development of which a few key examples will be discussed in the following paragraphs.

IGF-1R is one of the first tyrosine kinase found to be associated with liver cancer. An increase in the ligand, Insulin-like growth factor 2 (IGF-2), as well as the receptor, was found in cirrhosis as well as in HCC. Many biochemical studies have characterized the role of IGF-IR in hepatocarcinogenesis and its control over downstream cell cycling and anti-apoptotic pathways in HCC models [33, 34]. Within the last decade, efforts at developing targeted inhibitors have also demonstrated promising results on HCC. In 2006, a small molecule inhibitor, NVP-AEW541 was able to suppress growth and cell cycling in HCC cell line [35]. Other molecules as well as therapeutic monoclonal antibodies are now in various stages of pre-clinical and clinical development.

Focal adhesion kinase (FAK) is a non-receptor tyrosine kinase that was shown to be upregulated in HCC. In a recent study, FAK, together with a microRNA-151, were found to act synergistically to enhance cell motility and spreading in HCC. This controls the rate and extent of intra-hepatic metastasis of HCC, a major cause of mortality for the disease [36]. Separately, FAK activation has also been linked to

HBV gene product, HBx, and hence may be able pivotal point for pharmacological intervention against HBV-infected HCC [37].

Fibroblast growth factor receptor 4 (FGFR4) is constitutively and highly expressed in liver, hence suggesting its physiological function in the liver such as bile acid synthesis. Therefore, it is conceivable that perturbation of its signaling may result in pathological consequences. Recently, our group has found that approximately one-third of HCC patients demonstrated an elevation of FGFR4 in the tumor as compared to the adjacent normal tissue. Furthermore, there is an association between a frequently-occurring polymorphism, FGFR4-G388R, and an increased AFP levels in such patients. Inhibition of FGFR4 via both small molecule inhibitor and siRNA approaches suppressed cell proliferation and AFP production in HCC cell lines. These findings support the role of FGFR4 in HCC progression of which further investigation is underway [38].

Beside these, there are several other tyrosine kinases found to be modulated either by overexpression, or the acquisition of germ-line or somatic mutations in HCC. Theses associations prompted mechanistic work to establish causality as well as to investigate their potential as drug targets. Some additional examples include MET, PYK and many others. Table 1 below summarizes the tyrosine kinases that have been reported to be linked to HCC based on current literatures.

Table 1 Tyrosine kinases that have been implicated in HCC

Gene	Description	References
AXL	Preferentially expressed in HCC by parallel hybridization	[39, 40]
EphA1	Silencing of EphA1 exhibits anti-angiogenic and anti-tumor effect in HCC	[41]
EphB2	Upregulated 9-fold in HCC	[42]
FAK	mRNA expression correlated with tumor size, AFP, disease progression; Independent marker for prognosis; Activation by HBx	[37, 43]

FGFR3	Associated with poor tumor differentiation and increased nuclear grade	[44]
FGFR4	Preferentially expressed in HCC by parallel hybridization	[38, 39]
HER 2	Increased expression and association with HBx	[45]
HER3	Elevated HER3 and cognate partners by microarray; Associated with disease progression	[46, 47]
IGF-1R	Critical for malignant transformation in HCC	[33, 48]
JAK1	HBx activates JAK1 through direct protein interaction	[49]
JAK3	Higher basal level detected in HepG2	[50]
Kit	Expression in oval cells of most HCC of HBV-origin	[51-53]
MET	Mutations associated with accelerated carcinogenesis in childhood HCC: T1191I, K1262R, M1268I; Met silencing inhibited HCC growth in vivo	[54, 55]
PDGFR-α	Overexpression in vascular endothelium of highly metastatic HCC	[56]
PDGFR-β	Preferentially expressed in HCC by parallel hybridization	[39]
Pyk2	Contributes to tumor metastasis	[57, 58]
RON	Unregulated RON and MET expression associated with HCC	[59]
Src	Activation of Src in early HCC	[60]
TIE2	Overexpressed in neovascular endothelium of most HCC. More recently, Ang-1 and -2 are also shown to be upregulated	[61]
VEGFR1/2	Upregulated in EC of HCC	[62]
VEGFR3	Overexpression of VEGFR3-short after HBx stable transfection in HepG2 cells. VEGFR3-s correlate with survival in patients	[63]

This table is a compilation of tyrosine kinases reported to be associated in HCC based on a data-mining of literatures catalogued by PubMed (National Center for Biotechnology Information, NIH, USA). The reported genes are listed alphabetically and their corresponding references are cited.

Some tyrosine kinases inhibitors are already in various stages of clinical trials for HCC. For example, erlotinib and gefitinib, both EGFR inhibitors are in phase II or phase III clinical trials. Lapatinib, that target Her-2/neu, is now in phase II or phase III clinical trials. Sunitinib, another multi-kinase targeting inhibitor like sorafenib was also tested in HCC studies. Table 2 summarizes some of the ongoing trials involving tyrosine kinases. Currently, these clinical trials yielded mixed results. In an open-label phase II clinical study on sunitinib conducted on 37 patients, sunitinib showed severe hematological side effects and only one patient showed partial response [64]. Another phase II study on brivanib, an fibroblast growth factor

receptor 1 (FGFR1) and vascular endothelial growth factor receptor 2 (VEGFR2) inhibitor, with 55 unresectable metastatic tumors, revealed that almost half of the patients achieved stable disease or response (i.e. complete or partial response) with little use-limiting toxicities [65]. Several other clinical trials for various agents displayed only marginal responses [66, 67]. Clearly, more effort is required to understand the specific roles of different tyrosine kinases in HCC in order to better treat the disease using such pharmacological agents.

Table 2 Tyrosine kinase inhibitors in clinical trials for HCC treatment

Drug	Sponsor	Targets	Country	Phase
Sorafenib*	Bayer	VEGFR, PDGFR, Kit, Flt3	Multi-national	Approved
Gefitinib	Multiple	EGFR	Multi-national	II
Sunitinib	Multiple	VEGFR, PDGFR, Kit, Flt3	Multi-national	II
Bevacizumab/Erlotinib	Multiple	EFGR	US	II
Fostamatinib	NCI	Syk	US	II
Dasatinib	NCI	Bcr-Abl, Src	US	I/II
TSU-68	Taiho	VEGFR, FGFR, PDGFR	Japan	I/II
Pazopanib	GSK	VEGFR, PDGFR, c-kit	Multi-national	I
Lapatinib	NCI	EGFR, HER2	US	II
ABT-869	Abbott	VEGFR, PDGFR	Multi-national	II
Brivanib	BMS	VEGFR2, FGFR1	Multi-national	II/III
Cediranib	AstraZeneca	VEGFRs	US	I/II
Dovitinib	Novartis	FGFR/VEGFR/ PDGFR	Multi-national	II
Foretinib	GSK	MET/VEGFR	Asia	I/II

This table summarizes the various tyrosine kinase inhibitors clinical trials that are ongoing or completed for HCC. The data is obtained by mining the information from http://www.clinicaltrials.gov, as well as the review article by Huynh H, 2010 [68].

1.7 Tyro3

One of the subfamily of tyrosine kinases of growing interest to cancer research is the TAM receptor family. There are three members in TAM family, including Tyro3, Axl and Mer. Axl is the most well studied member in this family and has been found to be involved in many cancer types, including HCC [39]. It was

indicated that AXL is a mediator of Yes-associated Protein (YAP)-dependent oncogenic activities and implicates it as a potential therapeutic target for HCC [40]. In renal cancer cell lines, knockdown of Axl could influence cell phenotypes such as cell viability, apoptosis, etc. Meanwhile, expression changes of Axl could also influence cell cycle, such as prolonging G0/G1 period [69]. Separately, the other family member, Mer has been found to play roles in neuronal development [70]. The TAM receptor family exhibit significant sequence and structural similarities. For example, they all possess two extracellular Immunoglobulin domains, two fibronectin-III (FNIII) domains and one intracellular kinase domain. The extracellular segment embodies a receptor site for the binding of ligands to initiate the kinase signaling cascade. Protein S and Gas6 are specific ligands with high affinity for Tyro3, Axl and Mer. By binding to Protein S or Gas6, the TAM family members undergo dimerization, phosphorylation and/or glycosylation, which will then be transmitted as an intracellular signal for the activation of downstream cellular signaling.

Figure 1 Domain organization of Tyro3, Axl and Mer

TAM family consists of two extracellular immunoglobulin-like domains, two Fibronectin III domains, a single-pass transmembrane (TM) region, and one intracellular kinase domain [70].

Tyro3 is the least characterized member in the TAM family. Its complete sequence was first reported in 1993 [71]. In 1995, it potential role as an oncogene was first characterized by Nobel laureate Prof Harold Varmus. His group demonstrated the overexpression of Tyro3 in mammary tumors in rodents and the consequence of ligand-independent activation [72, 73]. Tyro3 is also named as Sky,

Rse and DTK. To date, Tyro3 has been found to play roles in melanoma and lung carcinoma based on its upregulation in these malignancies [74, 75]. However, the mechanism for its involvement is still under investigation.

The downstream of Tyro3 activation has been investigated in some studies, but it is still poorly understood. A number of proteins that potentially interact with Tyro3 were identified, including p85 β-subunit of PI3K, protein phosphatase 1(PP1), and RanBPM [76, 77]. It was demonstrated that ligand stimulation of an EGFR/Tyro3 chimera induces phosphorylation of Tyro3 and an activation of PI3K, and Akt, which resulted in a transformed phenotype. In NIH3T3 cells which express endogenous Tyro3, phosphorylation of ERK1/2 was increased by Gas6 stimulation [78]. Gas6 stimulation also upregulated the phosphorylation of ERK1/2 in mouse osteoclasts, which resulted in bone resorption [79]. Co-immunoprecipitation of Tyro3 transiently expressed in COS cells revealed a potential interaction with a phosphorylated SFK, but it remains unknown which SFK(s) (Src, Yes, and/or Fyn) interact with Tyro3 [80]. Many downstream effector genes of these pathways remain elusive, and see is to full complement of the biological effect mediated by Tyro3. As further efforts are needed to clarify these mechanistic details, the available information will serve as useful starting points for us to explore the subcellular changes upon perturbation of Tyro3 in HCC models.

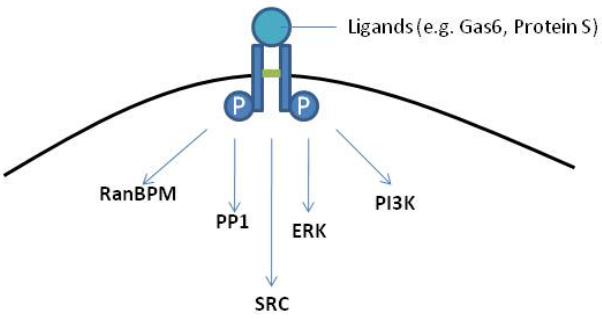


Figure 2 Signaling pathways Tyro3 has been reported to mediate (Adapted from [8])

Currently, Tyro3 signaling pathways mediate platelet aggregation, cell transformation, and osteoclastic bone resorption. The signaling molecules indicated in Figure 2 have been shown to associate with Tyro3 through either a direct or indirect interaction. Phosphorylation of Tyro3 at specific residues and their biological consequences remains uncharacterized [70].

2.0 HYPOTHESIS AND OBJECTIVES

Hepatocellular carcinoma (HCC) is the third leading cause of cancer death worldwide.

Yet, the understanding of its disease progression and options for treatment is still quite limited. Therefore, studying the mechanism underlying HCC development and progression is of great importance to establish new and better treatments for HCC that will benefit millions of HCC patients in the world [81]. In 2007, the newly approved tyrosine kinase inhibitor sorafenib represents a big breakthrough in HCC treatment. The unprecedented success of this drug supports identifying new tyrosine kinases as potential drug targets to help discover more molecular targeted inhibitors for HCC treatment. As there is limited number of tyrosine kinases reported to be involved in HCC development, we envision that there may still be a number of some tyrosine kinases that have not been extensively studied in this context. Preliminary results established within our group (unpublished data) suggested that Tyro3 could be one of these tyrosine kinases as we found Tyro3 phosphorylation to be significantly inhibited and resulted in the alteration of HCC phenotype in cell culture model systems. Furthermore, silencing of Tyro3 in the same cell culture model resulted in a reduced sensitivity for the cells towards the inhibitor. Hence, the finding suggests Tyro3 signaling may be critical in the effect of the inhibitor, and thereby a target worth investigating with greater depth. Tyro3 belongs to TAM (which also include AXL and MER tyrosine kinases) family, which has been found to play role in melanoma and hematological tumors [75, 82]. We speculate that Tyro3 may play important roles in other cancer types which may even include HCC.

As Tyro3 has not been characterized in HCC development before, it is meaningful to investigate its oncogenic potential using carefully designed models using of both *in vitro* and *in vivo* systems.

Therefore, the over-riding hypothesis of this research work is that the tyrosine kinase Tyro3 can play important roles in HCC progression such as cell viability. Elevated Tyro3 expression in the tumor versus adjacent normal tissue will have significant correlation with clinical data of HCC patients. These *in vivo* findings will shape subsequent *in vitro* validation work to establish detailed mechanism for its effect on HCC.

To test this hypothesis, the following aims have been crafted:

1. To determine the expression of Tyro3 in patient samples by RT-PCR, in order to find whether Tyro3 is overexpressed in HCC patients, through comparing the expression of Tyro3 in tumor tissues with its expression in normal tissues.
2. To investigate the correlation between Tyro3 expression and patient clinico-pathological parameters such as AFP level, AST level, age, gender, etiology, survival time, tumor size and multiplicity, etc.
3. To compare the expression of Tyro3 in different HCC cell lines by RT-PCR in order to develop a suitable Tyro3-silencing system for subsequent mechanistic investigations.
4. To compare the effect of Tyro3 silencing on cancer phenotypes such as cell proliferation.

3.0 MATERIALS AND METHODS

3.1 Cell Culture

Hep3B cell line was obtained from American Type Culture Collection (ATCC) (Manassas, VA, USA). Hep3B was maintained in Minimal Essential Medium (MEM) medium supplemented with 10% fetal bovine serum (FBS), sodium pyruvate (1 mM) and non-essential amino acids (100X). Cells were passaged every 4-5 days when they have reached 70% confluency in tissue culture dishes. To passage cells, medium in the dish was aspirated and 5 ml of pre-warmed phosphate-buffered saline (PBS) was added into the dish to rinse and wash the cells, and then the PBS was removed and 5 ml of 0.5% trypsin was added into the dish, incubated at 37 ºC for about 5 min. When the cells became round and detached from each other, trypsin was removed and cells were washed out with 5 ml of medium to inactivate the trypsin. The suspended cells were collected by centrifuging for 3 min at the speed of around 1,000 rpm. After which, cells were resuspended in appropriate volumes for subsequent seeding into new tissue culture dishes for experiments or cell maintenance. Cells were maintained at 37 °C in 5% CO_2. Unless stated otherwise, all cell culture reagents were obtained from Invitrogen (Life Technologies, Grand Island, NY).

Huh7, HepG2 and SK-Hep1 were also cultured to compare Tyro3 expression in different cell lines at the translational level. These cell lines were kind gifts from Prof Axel Ullrich's lab at Max-Planck Institute for Biochemistry, Martinsried, Germany. The medium used to culture Huh7 was DMEM with 10% serum, containing 1mM sodium pyruvate; and that for HepG2 and Sk-Hep1 was

MEM with 10% serum, 100x Non-essential Amino Acids (NEAA) and 1 mM sodium pyruvate.

3.2 HCC sample preparation

Total RNA was isolated from paired normal and HCC liver tissues from HCC patients (n=57) at the National University Hospital (Singapore) as previously described. These samples were obtained through collaborating with Prof Lim Seng Gee and complete with appropriate approval from the Institutional Review Board from the NUH. cDNAs were synthesized from 2 µg RNA using Superscript III reverse transcriptase kit (Invitrogen, Singapore), performed according to manufacturer's instructions and as briefly described in a later section.

3.3 Harvesting cells

After cell culture, the cells in dishes or 6-well plate were harvested. The cells were firstly washed with ice-cold PBS and collected by scraping. Cell pellets were collected by centrifuging at 6000 rpm for 1 min at 4 °C. For longer term storage, cell pellets were kept at -80 ^{o}C or otherwise subjected to cell lysis immediately.

3.4 Total RNA isolation

Total RNA was extracted using RNeasy kit from Qiagen (Singapore) with modified protocols as described below. For every 10 million cells, 1 ml of TriZol reagent (Invitrogen, Life Technologies, Grand Island, NY) was added into the tube which contained cell pellet. Cell pellet was homogenized by pipetting. 200 µl Chloroform (Sigma Aldrich, Saint Louis, MO, USA) was added and the tube was

vortexed for 15 sec. The tube was incubated on ice for 2-3 min and then spun at 10,000 rpm for 15 min at 4 °C. The clear aqueous phase was transferred into a new tube, and an equivolume of 70% ethanol (around 0.5 ml) was added into the new tube. 700 µl of the sample was transferred into RNeasy Mini column, and subsequent RNA isolation procedure was performed according to manufacturer's instruction. The concentration of total RNA was determined by NanoDrop (Thermo Scientific, Wilmington, DE, USA). The 260/280 nm absorbance ratio of the total RNA was maintained between 1.9 and 2.1.

3.5 cDNA synthesis

A system of 10 µl containing specific amount of total RNA, primer, dNTP mix (10mM), diethylpyrocarbonate (DEPC)-treated water was prepared. The reaction mix was incubated at 65 °C for 5 min, and at least at 4 °C for 5 min for the annealing of the Oligo-dT primers to the mRNAs. After that, the tubes were taken out and put on ice, and 10 µl of synthesis mix was added in the system. The synthesis mix contained 2.0 µl of 10×buffer, 4 µl of $MgCl_2$ (25mM), 2µl Dithiothreitol (DTT) (0.1M), 1µl RNase OUT (40U/µl), 1µl SuperScript III RT (200 U/µl). Then 10 µl of synthesis mix was added into the previous reaction mix, which was then incubated for 50 min at 50 °C and cooled to 4 °C. After that, 1 µl of RNase H was added and incubated at 37 °C for 20 min. The tubes were stored at -20 °C until further usage.

3.6 Primer design and gel electrophoresis

Primers were purchased from 1st BASE (Singapore). Two primers have been optimized for further experiments. The primers were designed using Primer 3

software (version 4.0, http://frodo.wi.mit.edu/primer3/). Forward primer for Tyro3 was 5'-CGGTAGAAGGTGTGCCATTT-3', reverse primer 5'-TGGGTCACCCCTGTTACATT-3'. GAPDH was used as reference gene. The sequence of forward primer for GAPDH was 5'-ATGTTCGTCATGGGTGTGAA-3', the sequence of reverse primer for GAPDH was 5'-TGTGGTCATGAGTCCTTCCA-3'. To test the suitability of primers for real-time polymerase chain reaction (PCR), PCR amplifications were performed with KOD hot start polymerase (Novagen, Madison, WI). PCR was carried out in 50 µl reaction volumes containing 2 µg cDNA templates, 2 mM dNTPS, 25 mM $MgSO_4$, 10×Hot start buffer and 10 µM forward and reverse primers. SYBR®-safe (Invitrogen, Singapore) was used to stain DNA bands. 50× Tris- acetate- EDTA (TAE) buffer was prepared with 2.42 g/ml Tris, 57.1% (v/v) acetate and 0.05 mol/L ethylenediaminetetraacetic acid (EDTA). Hot start polymerase from MERCK (Darmstadt, Germany) was used to amplify DNA. The PCR thermal cycling condition was: hold in 50 ºC for 10 min; for each cycle, denature in 85 ºC for 15 sec; anneal in 60º for 1 min and cycled 38 times. Next amplified DNA was examined by gel-electrophoresis using 1.5% agarose gel. The voltage for electrophoresis was set at 60-100V and left to run for 30 min. After gel electrophoresis, the gel was stained in Sybr-safe (Invitrogen) reagent for around 30 min and was visualized under UV illumination. Single band at the expected size indicates the specificity for the targeted amplicon.

3.7 RT-PCR to detect Tyro3 expression on transcriptional level

Quantitative real-time PCR (qRT-PCR) was performed in triplicates on the ABI7500 Fast Real-Time PCR system (Applied Biosystems, Foster City, CA). Each reaction contained 4µl synthesized cDNA, 2 µl each of 10 µM forward and

reverse primers, 2 µl nuclease-free water and 10 µl Power SYBR Green PCR Master Mix (Applied Biosystems). The cycle program for quantitative real-time was hold in 50 ºC for 10 min; for each cycle, denature in 85 ºC for 15 sec; anneal in 60 ºC for 1 min for a total of 40 cycles. Gene expression was analyzed with the $2^{-\Delta\Delta Ct}$ formula after normalizing to GAPDH. ΔCt=Ct(Tyro3)-Ct (GAPDH), ΔΔCt=ΔCt(tumor)-ΔCt(normal).

3.8 Harvesting cells for western blot

To harvest cells for western blotting, they were first washed with ice cold PBS. Cell scraper was used to dislodge cells from its monolayer in culture. Cells were transferred into microcentrifuge tubes. Tubes were spun at 5000 rpm for 2 min and supernatant was removed. The cell pellet was subsequently lysed with a buffer (containing 50 mM HEPES, 150 mM NaCl, 1 mM EDTA, 10% glycerol, 1% Triton-X-100, 10 mM sodium pyrophosphate) containing protease and phosphatase inhibitors (10 mM sodium fluoride, 2 mM sodium orthovanadate, 1 mM phenylmethylsulfonylfluoride, 0.1 µg/ml aprotinin) by gentle mixing in a rotating platform for 30 min at 4ºC. To enhance the detection of Tyro3, a membrane bound protein, a separate lysis buffer consisting of 20 mM Tris HCl (pH 8.0), 137 mM NaCl, 10% glycerol, 1% Nonidet P-40 and 2 mM EDTA was also used. Unless stated otherwise, chemicals were obtained from Sigma. After lysing cells, the tubes were centrifuged at 13,000 rpm for 10 min.

After mixing for 30 min, cells were centrifuged at 13,000 rpm for 15 min. The supernatant was transferred into a new tube, and the supernatant was used to test the protein concentration. Protein concentrations were determined by bicinchoninic acid (BCA) method (Pierce, Rockford, IL). Standard curve was derived from varying

concentrations of albumin solution (2 mg/ml stock), which include 100, 200, 400, 600, 800, 1000, 1200 µg/ml. Water blank was determined as negative control. Quality of protein concentration determination is based on R value of standard curve close to 1.0. Then the concentration of unknown protein could be determined by standard curve.

3.9 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

For Western blotting, equal amounts of proteins prepared and quantified as described earlier were resolved with 7.5% SDS-PAGE. The gels generally consist of acrylamide, bisacrylamide, SDS and a Tris-Cl buffer with adjusted pH. 7.5% separating gel (20 ml) contains 9.8 ml H_2O, 5 ml 30% Bisacrylamide, 5 ml 4×Tris buffer with SDS, 80 µl 10% ammonium persulfate (APS), 32 µl N,N,N',N'-tetra methylene diamine (TEMED). The pH for stacking gel is 8.0. The pH for separating gel is 6.8. 10% APS (Sigma-Aldrich) and TEMED (Merck Pte. Ltd., Singapore) are added when the gel is to be polymerized. 30-50 µg protein samples were added into each well. Protein ladder was also loaded as reference. The voltage for SDS-PAGE was firstly set as 60 V; upon loading dye crossing into the separating gel, the voltage for SDS-PAGE was increased to 100 V.

3.10 Western Blot

Semi-dry transfer technique was used to transfer the protein in gel on polyvinylidene difluoride (PVDF) membrane. The current for semi-dry transfer was around 45 mA per membrane. Then the protein in gel was transferred onto membrane by semi-dry transfer. The time for semi-dry transfer is around 2 hours and 20 minutes.

After transfer, membranes were blocked with milk using PBS with 0.05% (v/v) Tween 20 and 5% (w/v) non-fat milk for about 2 hours at room temperature. After blocking, blots were incubated in respective antibodies diluted in PBS with 0.05% (v/v) Tween 20 and 5% (w/v) non-fat milk. Primary antibody for Tyro3 was from Santa Cruz Biotechnology (Santa Cruz, CA), beta-actin from Abcam (Cambridge, MA). Secondary antibodies were anti-mouse and anti-rabbit Horseradish Peroxidase (HRP)-conjugated antibodies (Pierce). Incubations were performed overnight on a shaker which was placed in refrigerator (around 4 °C). β-tubulin antibody was diluted for 1,000 times in 5% milk/phosphate-buffered saline with Tween 20 (PBST) and incubated for overnight. Secondary antibody was anti-rabbit secondary antibody (which was diluted for 10,000 times in 5% milk/PBST) for around 2 hours. Chemiluminescence substrate used was West Dura Extended (Pierce).

For some western blots, an alternative condition was applied to improve on the detection signals: the membranes were blocked in PBS with 0.05% (v/v) Tween 20 and 5% (w/v) for overnight at 4˚C. The blot containing Tyro3/β-actin was incubated in a tube containing anti-rabbit Tyro3 antibody (1000× in PBS with 0.05% (v/v) Tween 20 and 5% (w/v) non-fat milk) at room temperature for 4 hours. After washing, the membranes were probed with secondary antibody for 2 hours at room temperature and the detection method was described as shown above.

3.11 Knockdown of Tyro3 by siRNA

Tyro3 is silenced in cell culture models, which was performed using siRNA. siRNA which can target Tyro3 and scrambled siRNA were purchased from Dharmacon (Chicago, IL). The transfection reagent Oligofectamine was from Invitrogen. The sequence of siRNA targeting Tyro3 were shown as below. Tyro3

siRNA10: ACGCUGAGAUUUACAACUA. Tyro3 siRNA 11: GCGAUGAACUAAAGGAAAA. To perform Tyro3 silencing, cells were plated into 6-well plate one day before transfection. Approximately 100,000 cells were seeded per well. Transfection was performed when the cell reaches 60% confluent. 24 h after seeding, cells were transfected with 100 nM non-targeting control siRNA and *tyro3* siRNA using Oligofectamine reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Briefly, siRNA: Oligofectamine complexes were added to the serum-free medium and incubated for 5 hours. Tube A was consisted of 4 µl Oligofectamine and 30µl serum free medium, tube B was consisted of 5.2 µl siRNA and 160.8 µl medium. After incubation for 5 min, tube A was added into tube B to make the siRNA: Oligofectamine complexes. After incubation for 30 min, the complexes were added to the cells. Cells were then replenished with medium with 10% fetal calf serum (FCS) and incubated for another 72 hrs. The efficiency of siRNA knockdown was tested by RT-PCR and Western Blot. Any cell morphological changes were also monitored be observing for visual changes using light microscope.

3.12 Cell Titer-Glo

48h post-transfection with *tyro3* targeting siRNA, eight replicates (10,000 cells per/well) were seeded in 96-well plate. After incubating for 48 h, CellTiter-Glo luminescent assay (Promega, Madison, WI) was performed to measure Adenosine Triphosphate (ATP) content as a gauge of the viable cell count. Assay was performed according to manufacturer's instruction. Luminescence was detected with SpectraMax M5 (Molecular Devices) and results were expressed in relative luminescence unit (RLU) vs. scrambled controls.

3.13 Statistical analysis

Statistical analysis was performed for the analysis of RT-PCR results and to establish correlations between the expression of Tyro3 and clinico-pathological data. SPSS software was employed for most of such analysis. Results were considered significant when *p* value was below 0.05. T test, One-Way ANOVA and Chi-square were used to analyze the results. T-test and One-Way ANOVA were used to analyze the quantitative data. Chi-square was used to analyze qualitative data.

4.0 RESULTS

4.1 Overexpression of Tyro3 in HCC patients

57 pairs of HCC patient samples were obtained from our clinical collaborator at the National University Hospital (NUH). These samples were supplied in the form of total RNA extracted from both HCC tissue and the adjacent normal liver tissue from the patients [38]. A former research student in the group has previously synthesized cDNA from the total RNA samples (manuscript under review). Here, we quantified the expression of Tyro3 in the tumors by RT-PCR and normalizing the levels against the respective paired normal tissues. In our study, overexpression is defined as fold change between tumor and normal tissue of greater than 2-fold, whereas low expression is defined as the fold change between tumor and normal tissue of less than 0.5. Two-fold threshold provides a reasonably significant cut-off for transcript level changes which is commonly used as a benchmark for overexpression. We noted that about 50% of the patients exhibited over expression of Tyro3 in the tumor tissues. This also represents the biggest subgroup of the HCC patients in our study (Figure 3). As shown in Figure 4, the highest fold change detected was ~20-fold in one specific patient, and 7 out of 57 patients demonstrated overexpression of greater than 10-fold. Hence, this overexpression warrants further investigation of its significance to the disease.

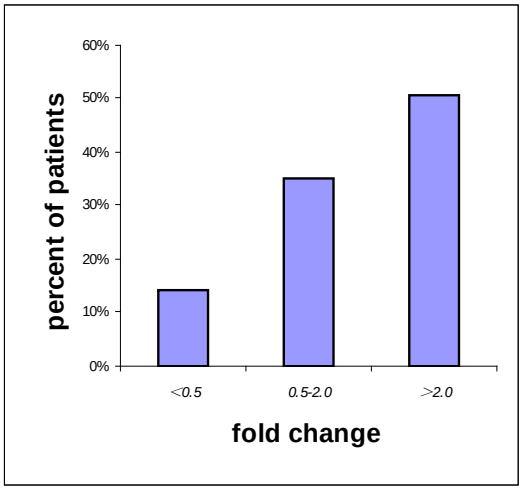


Figure 3 Overexpression of Tyro3 in patient samples.
Overexpression is defined as the fold change between tumor tissue and normal tissue of more than 2.0; medium expression is defined as fold change between tumor tissue and normal tissue in the range of 0.5-2.0.

4.2 Correlation of Tyro3 expression with clinical data

4.2.1 Correlation of Tyro3 expression with etiology

Given the frequent overexpression of Tyro3 mRNA in our patient samples, we investigated the correlation between Tyro3 expression profile and the clinic-pathological data of the patients. For these analyses, we define statistical significance as *p* value less than 0.05, using either chi-square or t-test analysis. The complete set of clinic-pathological data of the 57 patients is shown in Table 3. Specifically, it was shown that Tyro3 expression exhibited significant correlation with the etiology of the disease. Patients with HBV infection exhibited higher average Tyro3 overexpression in the tumors vs. normal tissue. Higher number of HBV infected patients were detected with two-fold or greater expression of Tyro3 compared to HCC patients without HBV infection based on chi square analysis. This information enables us to stratify our patients into low, medium and high subgroups. Table 4 summarized the expression of Tyro3 correlating with HBV infection.

Table 3 Clinico-pathological data (courtesy of Poh Wei Jie)

		Value	Distributions
Median age (95% CI)		58.07	(51.44, 61.71) 7-84
Gender	Female	11	19.64%
	Male	45	80.36%
Race	Chinese	39	69.64%
	Indian	5	8.93%
	Malay	2	3.57%
	Others (Indonesian, Pakistani, Myanmar)	9	16.07%
Etiology	HBV	41	73.21%
	HCV	3	5.36%
	Alcohol	1	1.79%
	Cryptogenic	11	19.64%
Outcome	Alive	18	32.14%
	Death	17	30.36%
	Recurrence	10	17.86%
	LOT	11	19.64%
Mean ALT(U/ml) (95% CI)*		58.26	(42.44,74.07) 14-343
Mean AST(U/ml) (95%CI)*		68.61	(52.06,85.16) 17-313
Mean AFP(U/ml) (95%CI)^		23030	(6348,39713)1.1-308590.0
	<400U/ml	30	54.55%
	>400U/ml	25	45.45%
Tumor multiplicity	Single tumor	34	60.71%
	Multiple tumors	22	39.29%
Tumor histology	Poor	15	26.79%
(Differentiation)	Moderate	33	58.93%
	Well to moderate	1	1.79%
	Well	6	10.71%
	Fibrolamellar variant	1	1.79%
Tumor diameters	8.54	(7.243, 9.828) 1.1-22.0	
Cirrhosis	Positive	37	66.07%
	Negative	19	33.93%

Vascular invasion	Yes	30	53.57%
	No	19	33.93%
	No report	7	12.50%

*n=51

^n=55

Table 4 Correlation between HBV infection and Tyro3 overexpression (ratio)

Etiology	Number of patients	Tyro3 expression			*p* value
		> 2 fold	0.5-2	<0.5	
HBV	41	20 (48.8%)	18 (43.9%)	3 (7.3%)	0.05
Non-HBV	15	5 (33.3%)	5 (33.3%)	5 (33.3%)	

High Tyro 3 expression is defined as 2-fold or more changes between tumor and normal tissue; medium expression is defined as the fold change in the range of 0.5-2.0; low expression is defined as the fold change between tumor tissue and normal tissue of less than 0.5

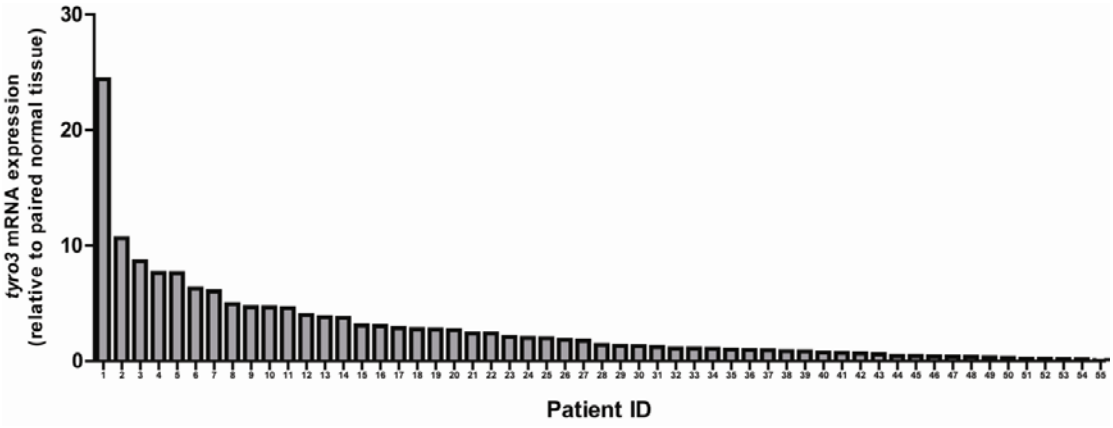


Figure 4 Fold change of Tyro3 expression between tumor tissue and normal tissue in individual patient.

Results indicated a total of 55 pairs of patient samples were tested. Overexpression is defined when the fold change between tumor tissue and normal tissue is above 2.0.

4.2.2 Correlation of Tyro3 expression with AFP level

AFP is an important clinical biomarker for HCC detection [83]. In about 60-70% of the patients, increased AFP correlates with disease severity [84]. Using the

clinical threshold of 500 ng/ml for high AFP expression, we found that patients with high AFP exhibited higher expression of Tyro3 with an average fold change of 5.6 compared to 2.5 in patients with lower AFP group. The results are shown in Table 5.

Table 5 Correlation between Tyro3 expression fold change and AFP level

AFP level (ng/ml)	Number of patients	Average fold change	*p* value
≤ 500	31	2.52±3.27	0.01
> 500	24	5.60±5.43	

At the clinical threshold of 500 ng/ml, patients with high AFP exhibited higher expression of Tyro3.

4.2.3 Correlation of Tyro3 expression with AST level

AST and ALT are clinical biomarkers in serum. The expression of AST and ALT correlates with hepatocellular damage and serves as an indirect indicator of HCC disease status. AST is an enzyme in the liver, when the liver function is damaged, AST will be released into the blood. Here we examine correlation if any between Tyro3 expression and AST in HCC patients. For the patient cohort with serum AST higher than clinical level of 40 IU, more than 50% patients demonstrated high Tyro3 expression in the tumor. Patients with AST level below 40 U/I have relatively lower ratio of Tyro3 overexpression. The results are shown in Table 6.

Table 6 Correlation between AST level and overexpression of Tyro3

AST level (U/I)	Number of patients	Tyro3 expression		*p* value
		High	Low	
>40	30	16(53.3%)	14(46.7%)	0.04
≤40	21	5(23.8%)	16(76.2%)	

At the clinical cutoff of 40 U/I, patients whose AST levels were above 40 U/I have higher ratio of Tyro3 overexpression than patients whose AST levels were below 40 U/I.

4.2.4 Correlation of ALT level with overexpression of Tyro3

Similar to AST, ALT is another important marker for liver function and elevated levels correlates with liver injury. Our results demonstrated that patients with ALT level higher than clinical level of 35 U/I, the ratio of Tyro3 over expression in HCC patients were also significantly higher compared to patients whose ALT level is below 35 U/I. The results are shown in Table 7.

Table 7 Correlation between Tyro3 over expression and ALT level

ALT level (U/I)	Number of patients	Tyro3 expression		p value
		High	Low	
>35	31	17(54.8%)	14(45.2%)	0.01
≤35	20	4(20.0%)	16(80.0%)	

At the clinical cutoff of 35U/I, patients whose ALT levels were above 35 U/I have higher ratio of Tyro3 overexpression than patients whose ALT levels were below 35 U/I.

4.2.5 Correlation between Tyro3 over expression and tumor size

A way to measure tumor aggressiveness is by measuring the tumor growth. Here, we found that Tyro3 overexpression in HCC patients significantly correlated with tumor size. The tumor size is represented by the tumor diameter at its longest dimension. When Tyro3 is overexpressed, the average tumor size is significantly bigger than that of patients without Tyro3 overexpression. 51% of patients' tumors with tumor diameter of more than 3 cm showed overexpression of Tyro3 with *p* value less than 0.05. Whereas, none of the patients with tumor diameter lesser than 3 cm overexpresses Tyro3.

Table 8 Correlation between tumor size and overexpression of Tyro3

Tumor size (cm)	Number of patients	Tyro3 expression		*p* value
		High	Low	
>3	49	25 (51.0%)	24 (49.0%)	0.02
≤3	7	0 (0%)	7 (100%)	

At the cutoff of 3cm, patients with tumors more than 3 cm have higher ratio of Tyro3 overexpression than patients whose tumors were smaller than 3 cm.

4.3 Tyro3 expression in different liver cancer cell lines

Based on the strong correlation between Tyro3 expression and various clinic-pathological characteristics in HCC, we embarked on subsequent *in vitro* investigation to validate the *in vivo* observation as well as to gain mechanistic insight on the role of Tyro3 in this disease. Our first step was to select suitable HCC cell lines as our experimental models. Hence, we surveyed the expression of Tyro3 expression across different HCC cell lines, which include HepG2, Hep3B, Primary Liver Cancer (PLC)/PRF/5, Sk-Hep1, Huh7, as well as non-cancerous liver cell lines THLE2 and Hs1.Li. We tested the Tyro3 expression by RT-PCR and immunoblotting. Our results demonstrated that most liver cancer cell lines overexpressed Tyro3 when comparing with THLE2 and Hs1.Li cell lines. The highest expression of Tyro3 was found in Hep3B, which is a cell line carrying HBV infection (Table 9). The second highest expression of Tyro3 was found in HepG2 and the third highest was found in Huh7. Subsequently, western blotting confirmed the transcriptional expression results where Hep3B was also found to be the highest Tyro3 protein expressing cells (Figure 6). Huh7 has the next highest protein expression besides Hep3B. As Tyro3 is expressed highly in Hep3B cell line, it is meaningful to study the role of Tyro3 in HCC progression using Hep3B cell line.

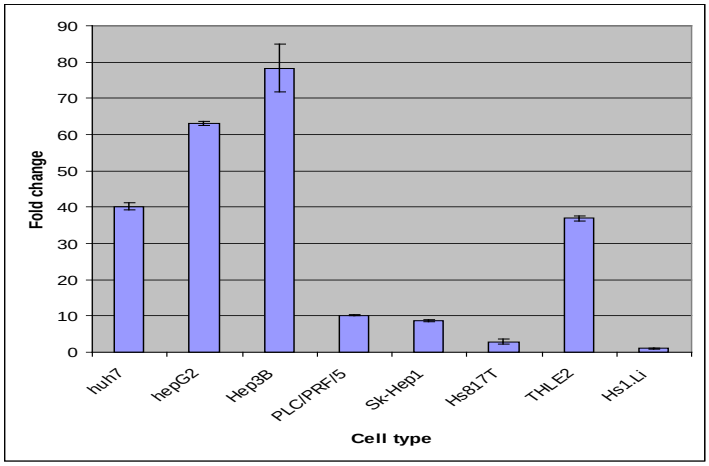
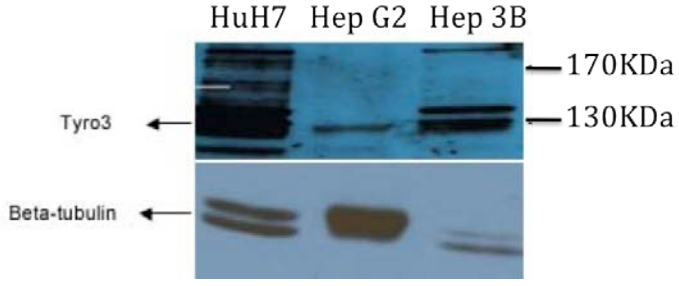


Figure 5 Comparison of Tyro3 expression in different liver cancer cell lines on transcriptional level.

RT-PCR was performed to test the expression of Tyro3 in different cell lines. The expression of Tyro3 was normalized against respective GAPDH as housekeeping control and fold-change normalized against Hs1.Li as normal hepatocyte cell line. Each fold change is expressed as the mean of duplicate runs.

Table 9 Hepatitis B status of HCC cell lines used

Cell Line	Hepatitis B infection status
Huh7	Negative
HepG2	Negative
Hep3B	Positive
PLC/PRF/5	Positive
SK-Hep1	Negative
THLE2	Negative



Ratio (Tyro3/β-tubulin): 1.38 0.24 3.46

Figure 6 Tyro3 expressions in Huh7, HepG2 and Hep3B liver cancer cell lines.

Whole cell lysate were harvested from Hep3B, Huh7 and HepG2 cells. 50 μg protein lysate was loaded and resolved with 7.5% SDS-PAGE gel. Western blot was performed using anti-Tyro3 (Santa Cruz) and anti-β-tubulin as loading control. The

expression was quantified densitometrically (Image J) and presented as a ratio of Tyro3:β-tubulin.

4.4 Knockdown of Tyro3 in Hep3B cell line

To study the effect of Tyro3 on cell growth properties of HCC cells, knockdown of Tyro3 was performed to silence Tyro3 expression. Tyro3 is knocked down in high-expression cell line, Hep3B cell line using siRNA either si10 or si11. Here, we found 80-90% of Tyro3 was silenced using RT-PCR as shown in Figure 7. Hence, the protocol was adopted for subsequent phenotype-based assay to validate the effect of Tyro3.

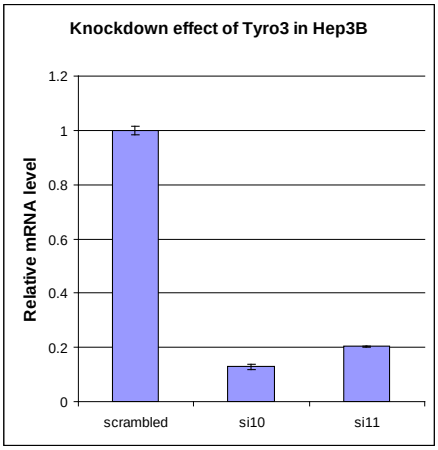


Figure 7 Tyro3 is silenced in Hep3B cell line.
Hep3B cells were transfected with either scrambled siRNA, si10 or si11, using oligofectamine according to manufacturer's instruction. cDNAs we re prepared from transfected cells after 48hrs, Tyro3 transcript was assessed via RT-PCR.

4.5 Effect of Tyro3 silencing on Hep3B cell viability

Cell Titer Glo was used to determine the impact of Tyro3 silencing on cancer phenotype. As a specific indicator of intracellular ATP levels, the assay provides an early indication if Tyro3 signaling may alter important regulators of cell proliferation and cell viability machineries after Tyro3 knockdown. After 72 h of

silencing, we found both siRNA constructs effectively reduced cellular ATP levels to about 60% of control as shown in Figure 8. Our data indicates that Tyro3 plays an important role in maintaining cell viability of Hep3B cells.

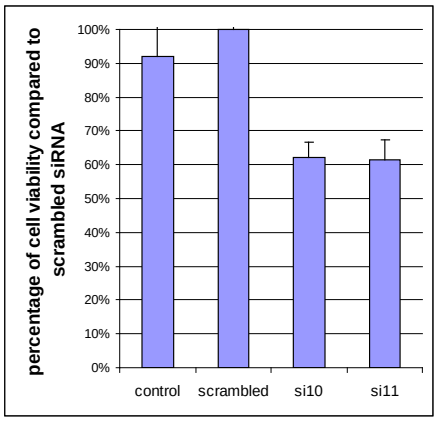


Figure 8 Silencing of Tyro3 reduces cell viability.
Hep3B cells were treated with scrambled siRNA, si10 or si11 for 48 hours later. Cell viability was tested using Cell Titer-Glo. Results are shown as percentage of cell viability compared to scrambled siRNA.

5.0 DISCUSSION

The value of molecular targeted therapy to cancer treatment is about finding a unique signaling mechanism specific to the cancer that may be accountable for the manifestation of the disease. The pivotal role of Bcr-Ab1 translocation in chronic myelogenic leukemia, activating mutation of EGFR in non-small cell lung cancer, as well as the amplification of HER2 in breast carcinoma are some of the notable examples that energized widespread efforts to consider this relationship in other cancer types [85]. Among the molecular targets identified to date, tyrosine kinase family has found greatest success with 9 agents that have obtained FDA approval as of 2010 (http://www.fda.gov). However, a reciprocal challenge to the specificity of tyrosine kinase mediated cell signaling is that every cancer tissue type may also utilize a different subset of kinases for its growth and maintenance. Hence, an effective application of tyrosine kinase inhibitors must involve an in-depth understanding of a particular tyrosine kinase and its role towards the malignancy of interest. In the case of HCC, a specific target remains elusive. Associations have been made between the disease and various tyrosine kinases but none appear to be single-handedly accountable for most of the observations. In this study, we have focused on a relatively uncharacterized tyrosine kinase, Tyro3, in the context of liver cancer, based on promising preliminary study suggesting its effect on HCC cell line.

Accordingly, we have chosen to embark on a search for correlations between Tyro3 expression and clinical data, as an entry point to this study. We envisioned that any link between increased Tyro3 expression and disease manifestation will provide important basis and direction for subsequent elucidation of

mechanism as well as further characterization of Tyro3 as a drug target. Firstly, the survey of 57 HCC patients revealed that almost half of them exhibit a significant elevation of Tyro3 transcript expression in tumor as compared to the adjacent normal tissue. This finding suggests the possibility that increased Tyro3 may be implicated in the transition of HCC from the normal to the tumor state. This is an important discovery that provides the early basis to support a further investigation into this area. The elevation observed in our study parallels the upregulation of AXL in HCC, another member of the TAM receptor family, which was also reported previously [39]. Furthermore, it mirrors the upregulation of Tyro3 seen in other malignancies such as melanoma and lung carcinoma [74, 75].

In order to associate the upregulation of Tyro3 in HCC to disease outcome, we performed a series of statistical analyses to detect correlation between expression and clinic-pathological states. We found that increased Tyro3 expression correlated well with higher levels of liver injury markers such as ALT and AST. These markers are common indicators of hepatocellular damage, which are also important sequelae of HCC. Therefore, the association with high Tyro3 may indicate a possible role to the progression of the disease. This link warrants further investigation using *in vitro* model system, which will be discussed subsequently.

Besides hepatocellular dysfunction, Tyro3 exhibited strong correlation with a number of direct markers of HCC. Higher AFP levels were shown to be linked to higher levels of Tyro3. This was also accompanied by diagnoses of larger tumor size at the point of surgical resection. The exact role of AFP in HCC is not known. However, this is a serum protein secreted in about 60-70% of all HCC patients [84]. In these patients, the level of AFP correlates well with the severity of the disease. In another words, a patient with a high AFP level may suffer a worse prognosis than

another with lower AFP. On the other hand, a larger tumor underpins the immense growth potential of the cancerous cells contributing to the tumor mass. Therefore, the combination of both increased tumor size and higher Tyro3 levels suggest that Tyro3 may have a significant role in the disease.

An interesting observation we made was the connection between elevated Tyro3 and the HBV-carrier status of the patient. We found that a much larger percentage of HBV carriers demonstrated higher expression of Tyro3 in their tumors versus the adjacent normal liver tissue. This raises a specific question with regards to the role of Tyro3 signaling on the etiology of the disease. Since the late 1990s, it was reported that HBV DNA could integrate into host DNA near the domains of tyrosine kinases such as EGFR. HBV gene product, HBx, was also shown to affect the transactivation of EGFR [86]. This mechanism is believed to transmit hyperproliferative signals for cells via downstream ERK phosphorylation and increase cell cycling. Similar studies in HCV have also demonstrated the activation of ERK phosphorylation and an increase in the aggressiveness of the consequent HCC [87]. Since these founding works, other investigators have also reported the possible involvement of other tyrosine kinases whereby SRC kinase family and FAK were shown to be activated through a direct association with HBx protein [37, 88]. More recently, other studies have also alluded to an increase in the expression of proto-oncogenes such as c-KIT (a key receptor tyrosine kinase found to be linked to the pathogenesis of gastrointestinal stromal tumors (GIST) particularly among HBV carriers who progressed into chronic hepatitis, cirrhosis or HCC [52]. The major implication for these findings is that aberrant expression and activation of tyrosine kinase can play important role on the hepatocarcinogenesis, thereby making them potential drug targets for both therapeutic and prophylactic purposes. Our observation

of a positive correlation between increased Tyro3 expression and HBV-carrier status therefore deserves further characterization.

Clinical correlation studies provide the relevance and rationale that Tyro3 may have a role to play HCC. Yet, it is not clear whether the upregulation of Tyro3 is a cause or an effect of disease progression. For this reason, our principal approach to establish the relationship is to perform *in vitro* studies to investigate consequence of activating or silencing Tyro3 function. This will enable us to determine the functional role of Tyro3 in HCC as well as to gain mechanistic insights for the phenotype it may generate. To support this effort, we selected suitable HCC cell lines that express inherently high levels of Tyro3 as *in vitro* models. From our available HCC cell lines, we found Hep3B, a HBV-infected HCC cell line, to exhibit the highest level of Tyro3 expression. It is interesting to note that even from our limited pool of cell lines, we already observed stronger expression among the HBV-infected ones, similar to the trend observed from our clinical data. Hence, our data substantiates the growing evidence of high Tyro3 expression in cancer tissues as documented by Human Protein Atlas (http://www.proteinatlas.org) for protein expression, and the GNF SymAtlas (http://www.biogps.gnf.org) for mRNA expression.

To establish the functional consequence of Tyro3 in HCC cell line model, we performed gene silencing of Tyro3 using chemically synthesized siRNA. This approach allowed us to investigate the direct effect of Tyro3 silencing on cancer phenotype, of which we have chosen cell proliferation as a proof of concept. Very clearly, we observed a significant suppression of cell viability in response to Tyro3 knock-down, using intracellular ATP production as a surrogate marker for viability. Therefore, not only is Tyro3 contributing to cell survival in HCC cell line, this result

also suggests that Tyro3 may play a pivotal role among other tyrosine kinases that may also be concurrently active in this cell line. This outcome is a key consideration of suitability in molecular-targeted therapy. The protagonist should play a dominant role in maintaining the cancer phenotype so that its inhibition can be effective as therapy.

That said, our finding is at best, preliminary, in its current state. Further characterization is needed in a few directions. Firstly, we need to ensure that the effect seen in Hep3B is not peculiar in this cell line. The same study should be replicated in other HCC cell lines. Secondly, the effect of Tyro3 silencing on other cancer phenotypes should also be monitored. AFP secretion is a specific marker for HCC progression and the outcome of Tyro3 inhibition/silencing on its levels would suggest whether such therapy could alter the prognosis of the disease. Intra-hepatic and extra-hepatic metastases are common causes of death for HCC [89]. Recent breakthroughs in pharmacotherapy of HCC using tyrosine kinase inhibitors such as sorafenib are thought to involve anti-metastatic activities through blocking angiogenesis [90]. Therefore, examining the effect of Tyro3 on different aspects of metastasis (e.g. cellular invasion, epithelial-mesenchymal-transition, angiogenesis) should also be included as part of the repertoire of cancer phenotypes. Finally, biochemical investigation should be included to understand the cellular signaling perturbation as a result of Tyro3 silencing. The signaling cascade that propagates Tyro3 phosphorylation and activation has not been fully understood. The target genes of such pathways that may be specific to liver physiology should also be identified in order to pinpoint other sites of intervention to support pharmacotherapy. Some specific pathways to consider include examining PI3K/AKT and ERK/MAPK phosphorylation as principal downstream regulators of cancer phenotypes. The

potential interaction of between Tyro3 kinase signaling and the viral protein, HBx should also be studied to establish any mechanistic link that may account for the clinical correlation between Tyro3 expression and HBV infection in patients. Also, cross-talks in cell signaling between tyrosine kinases contribute to treatment resistance. Therefore, such biochemical understanding will help us to recognize other targets that should be inhibited concurrently to enhance treatment response.

In our subsequent investigations, we also believe that we can draw reference from the findings of Tyro3 in other cancer types. In melanoma, it has been reported that Tyro3 could induce Melanocyte-specific MITF (MITF-M) expression [75]. It was shown that silencing of Tyro3 by shRNA *in vitro* resulted in a decrease in cell number with a corresponding decrease in cells undergoing apoptosis. When Tyro3 is knocked down *in vivo*, tumor shrinkage was observed in a time-dependent manner. It is suggested that Tyro3 may play its role in melanoma development through signaling pathway that involves microphthalmia-associated transcription factor (MITF) and implicate this as a possible drug target for development.

On a separate note, Tyro3 perturbation may lead to a change in expression levels of Axl and Mer. It is possible that the change in expression of one member of the TAM family may result in expression changes of the other two members due to the redundancy of signaling. By knowing more about the mechanisms and their involvement in HCC progression of these tyrosine kinases, as well as exploring more tyrosine kinases that are related to HCC development, we can have a better understanding of HCC development. This will lead us to find more methods and drugs to treat HCC, which may contribute to overall HCC management.

The knowledge gap of Tyro3 signaling in cancer and liver physiology in general is immense. This project provides a timely model and an appropriate research question to address some of these issues in a more holistic manner. Overall, we hope that these endeavors will provide additional tools in the understanding and treatment of an otherwise problematic medical condition.

6.0 CONCLUSION AND FUTURE DIRECTIONS

Our project set out to investigate the role of tyrosine kinase Tyro3, a relatively uncharacterized tyrosine kinase in HCC. Using a representative resource of HCC patients' samples obtained from National University Hospital, we first reported a significant overexpression of Tyro3 in about half of the patient samples. Importantly, by correlating Tyro3 expression with clinical data, we found elevations in Tyro3 to correspond to individuals with concurrent HBV infection, higher levels of tumor marker AFP, as well as higher liver disease markers ALT and AST. These outcomes provide strong basis to support further *in vitro* work to elucidate the mechanistic role of Tyro3 in HCC disease progression. Accordingly, we surveyed a number of cell lines and selected a high Tyro3-expressing HCC line, Hep3B, as an *in vitro* model for investigation. We successfully silenced the expression of Tyro3 and found a significant effect of Tyro3 in maintaining the proliferation of Hep3B.

Therefore, this work provided critical preliminary data that implicate Tyro3 as a new tyrosine kinase target which may play crucial role in the pathogenesis and progression of HCC. Through this effort, we have also developed a set of tools that enables us to probe further into the specific role of Tyro3 in a relevant cell type.

Given the huge heterogeneity of the disease, we subscribe to the belief that Tyro3 may play a contributory role as part of the complex signaling that maintains the phenotype of HCC. However, the relative importance of its contribution with respect to other known oncogenic signaling pathways awaits further investigation. Therefore, with a better understanding of how Tyro3 participate in this

process, possible development of Tyro3 as a potential drug target or as an adjuvant therapy can be visited both objectively and systematically (Figure 9).

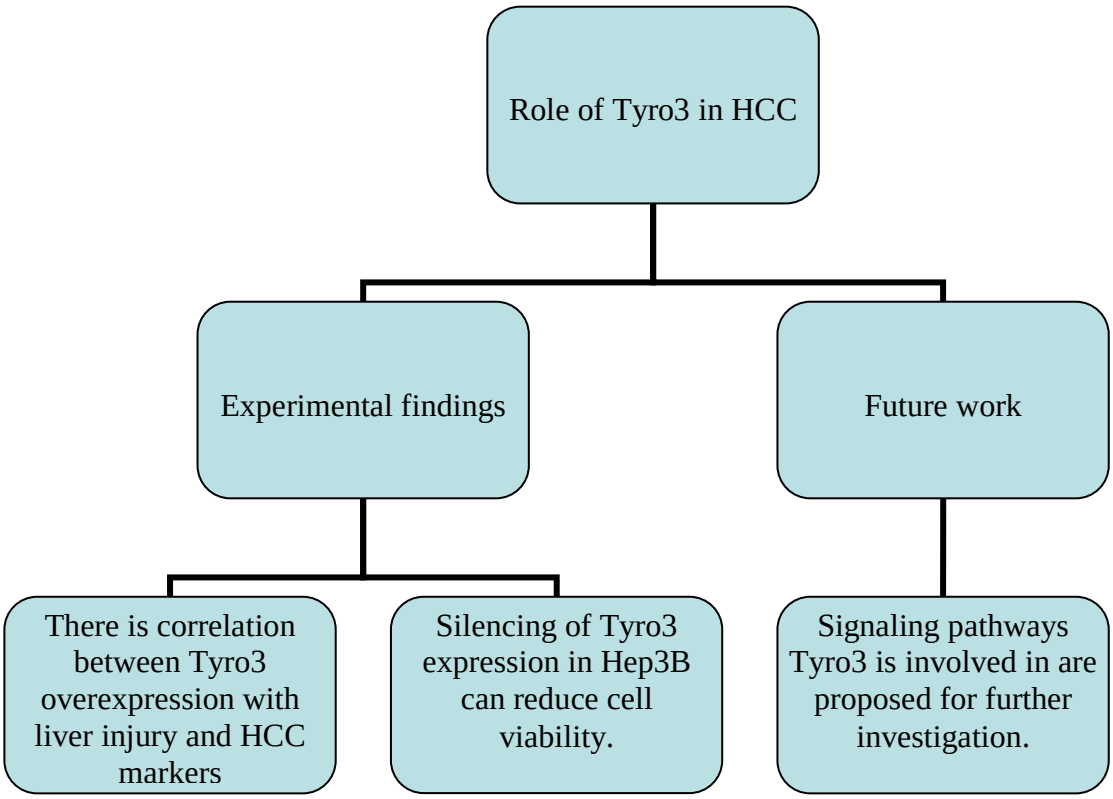


Figure 9 Experimental findings and future work

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